1-4252k

MYOCARDIAL GRAFTS AND CELLULAR
COMPOSITIONS USEFUL FOR SAME

BACKGROUND OF THE INVENTION

The present invention resides generally in the field of cardiology, and more particularly relates to stable myocardial grafts and methods and cellular compositions useful for achieving such grafts.

As further background, organ transplantation has been widely used to replace diseased, nonfunctional tissue. More recently, cellular transplantation to augment deficiencies in host tissue function has emerged as a potential therapeutic paradigm. One example of this approach is the well publicized use of fetal tissue in individuals with Parkinsonism (reviewed in (1), see reference list, infra.), where dopamine secretion from transplanted cells alleviates the deficiency in patients. In other studies, transplanted myoblasts from-uneffected siblings fused with endogenous myotubes in Duchenne's patients; importantly the grafted myotubes expressed wild-type dystrophin (2).

Despite their relevance in other areas, these earlier studies do not describe any cellular transplantation technology which can be succesfully aplied to the heart, where the ability to replace damaged myocardium would have obvious clinical relevance. Additionally, the use of intra-cardiac grafts to target the long-term expression of angiogenic factors and ionotropic peptides would be of therapeutic value for individuals with myocardial ischemia or congestive heart failure, respectively.

In light of this background there is a need for the development of cellular transplantation technology in the heart. Desirably, such technology would not only provide stable grafts in the heart but also enable the delivery of useful recombinant proteins or other molecules directly to the heart. The present invention addresses these needs.

SUMMARY OF THE INVENTION

The applicant has established cellular grafts in the myocardium which are viable long-term. Cardiomyocytes and skeletal myoblasts have been grafted directly into the myocardium of syngeneic animals. Viable grafts were detected at least one-half year post-implantation (the latest time point assayed). The presence of the grafts was not accompanied by overt cardiac arrhythmia, and the majority of the grafts were juxtaposed directly to the host myocardium and not encapsulated. It has thus been discovered that the myocardium can serve as a stable platform for cellular transplants. These transplants can be used for the local delivery of recombinant molecules to the heart and/or for replacing diseased tissue to supplement myocardial function.

Accordingly, one preferred embodiment of the invention provides a myocardial graft in an animal which includes a stable graft of skeletal myoblasts or cardiomyocytes incorporated in myocardial tissue of the animal.

Another preferred embodiment of the invention provides a method for forming a stable myocardial graft in an animal. The inventive method includes the step of introducing skeletal myoblasts or cardiomyocytes in myocardial tissue of the animal so as to form a stable myocardial graft. The cells can be conveniently introduced, for example, by injection.

Another preferred embodiment of the invention provides a method for delivering a recombinant molecule to myocardial tissue of an animal. This method includes the step of establishing a stable graft of skeletal myoblasts or cardiomyocytes incorporated in myocardial tissue of the

animal, wherein the myoblasts or cardiomyocytes deliver the recombinant molecule to the myocardial tissue. In this embodiment the myoblasts or cardiomyocytes will carry transgenes encoding the recombinant molecule.

Another preferred embodiment of the invention provides a cellular composition comprising a substantially homogeneous population of non-immortalized cardiomyocytes. This and other cell populations can be obtained utilizing a preferred inventive method that includes (i) transfecting embryonic stem cells to introduce a marker gene enabling selection of one cell lineage from other cell lineages resulting from differentiation of the stem cells, (iii) causing the stem cells to differentiate, and (iv) selecting said one cell lineage based on the marker gene. The cells used in and resulting from such methods also form a part of the present invention.

Still another preferred embodiment of the invention provides a non-human animal having a stable graft of skeletal myoblasts or cardiomyocytes incorporated in myocardial tissue of the animal.

The invention thus provides myocardial grafts, methods and cellular compositions useful for forming myocardial grafts, and animals which have the myocardial grafts. The grafts will find use both as a vehicle for delivering therapeutic substances such as recombinant proteins and other molecules, and as a means for replacing diseased tissue to supplement myocardial function. Cellular compositions of the invention can be used directly to prepare grafts, and will also be useful in screening drug substance effects on cardiomyocytes and for expressing and obtaining recombinant proteins. Grafted animals can be used, for example, to screen the effects of

recombinant molecules on the heart.

Thes and other objects and advantages of the invention will be be apparent from the following description.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 is a schematic diagram illustrating DNA used to generat MHC-nLAC transgenic mice in Example 3, _infra._

DESCRIPTION OF THE PREFERRED EMBODIMENT

For the purpos of promoting an understanding of the principles of the invention, reference will now be made to certain embodiments thereof and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations, further modifications and applications of the principles of the invention as illustrated herein being contemplated as would normally occur to one skilled in the art to which the invention relates.

As indicated above, the present invention provides stable myocardial grafts of skeletal myoblasts and/or cardiomyocytes. In this regard, as used herein the term "stable myocardial graft" is intended to mean a myocardial graft whose cells are viable for a period of at least about 2 weeks. Surprisingly, such stable grafts have been readily achieved in accordance with the invention, with preferred grafts having cells viable for six months or more. Myocardial grafts of the invention can thus provide for long-term delivery of recombinant proteins or other molecules to the heart and/or long-term supplementation of myocardial tissue.

The skeletal myoblasts and cardiomyocytes used in the invention can be obtained or isolated from any suitable source. Skeletal myoblasts, including for example C2C12 skeletal myoblasts, are available from public depositories such as the American Type Culture Collection (ATCC) (Rockville, Maryland). Skeletal myoblasts can also be isolated from skeletal muscle using techniques well known to the art and literature. Cardiomyocytes useful for the invention can be obtained using techniques described in the literature (3) or using methods described more

particularly in the Examples below. Briefly, one such
method involves digestion of heart tissues to obtain
cardiomyoctes.

Another method involves using an appropriate marker
to select specific cell lineages, such as cardiomyocytes,
from other cell lineages resulting from the
differentiation of embryonic stem cells (totipotent cell
lines derived from the inner cell mass of blastocysts as
described in (22)). The preferred method involves a
positive selection scheme. Thus, a marker gene, such as a
gene conferring antibiotic resistance (e.g. neomycin or
hygromycin), is introduced into the stem cells under
appropriate control such that expression of the gene
occurs only in the desired cell lineage. For example, the
marker gene can be under the control of a promoter which
is active only in the desired cell lineage. Upon
differentiation of the stem cells, the desired lineage is
then selected based upon the marker, e.g. by contacting
the mixed differentiated cells with the appropriate
antibiotic to which the desired lineage has been conferred
resistance. Cell lines other than the desired line will
thus be killed, and a substantially pure, homogeneous
population of the desired line can be recovered. In more
preferred methods, two markers are introduced into the
parent stem cells, one allowing selection of transfected
stem cells from non-transfected cells, and one allowing
selection of the desired cell lineage from other
lineages. A double positive selection scheme can thus be
used where each selectable marker confers antibiotic
resistance. Using this selection methodology, populations
comprised about 90% and even about 95-100% of the desired
cell lineage can be obtained, as demonstrated in the
Examples below.

To obtain grafts of the invention, the skeletal
myoblasts or cardiomyocytes will be introduced into the

myocardial tissue of a living animal such as a mammal.
The cells can be introduced in any suitable manner, but it
is preferred that the mode of introduction be as
non-invasive as possible. Thus, delivery of the cells by
injection, catheterization or similar means will be more
desired.

The resulting graft-bearing animals have exhibited
normal sinus rhythms, indicating that the graft per se, as
well as the graft-host myocardium border zone, does not
induce arrhythmias. This is in stark contrast to the
remodeling that frequently occurs following infarcts in
humans; the border zone of the infarct may give rise to
circus loops which result in clinically significant
arrhythmias (4, 5).

Grafts of the invention can be proliferative or
non-proliferative. For example, the AT-1 grafts
established in the specific Examples below are
proliferative. On the other hand, the skeletal
myoblast-derived grafts formed in the Examples are
non-proliferative, with the absence of tritiated thymidine
uptake demonstrating that the formation of stable
intra-cardiac grafts was not dependent upon sustained cell
proliferation.

Preferred grafts will be characterized by the
presence of direct intracellular coupling and the
formation of gap junctions between host and grafted
cells. Moreover, such grafts will not cause immune
response in the host, and will exhibit terminal
differentiation of grafted cells and a non-tumorogenic
nature.

Grafts of the invention are useful inter alia to
deliver therapeutic proteins and the like via secretion

from grafted cells, and to replace diseased or damaged tissue to supplement myocardial function. As examples of therapeutic protein deliveries, grafts may express angiogenic factors (as exemplified by basic and acidic Fibroblast Growth Factor; Transforming Growth Factor-Beta, Vascular Endothelial Growth Factor and Hepatocyte Growth Factor) to induce neovascularization. Similarly, grafts expressing neurotrophic agents near an infarcted region may be used to ameliorate the arrhythmogenesis associated with the border zone. These and many other candidate substances for targeted delivery to the heart will be apparent to those skilled in the area.

To promote a further understanding of the invention and its principles and advantages, the following specific Examples are provided. It will be understood that these Examples are illustrative, and not limiting, in nature.

EXAMPLE 1
Generation of Stable AT-1 Cardiomyocyte Grafts

A. METHODS

AT-1 Cell Culture and Myocardial Grafting Protocol. AT-1 cardiomyocytes were isolated from subcutaneous tumors by sequential collagenase digestion and cultured in PC-1 medium (Ventrex, Coon Rapids MN) containing 10% fetal calf serum as previously described in (6). Cells were labeled with 10 μM 8-chloromethyl-4,4-difluoro-1,3,5,7,- tetramethyl-4-bora-3a,4a-diazaindecene (BODIPY, Molecular Probes, Eugene OR) for 30 min at 37°C to facilitate localization of the injection site. Immediately before injection, cells were harvested with trypsin and collagenase, washed three times with serum-free PC-1 medium and directly injected into the ventricular myocardium of syngeneic B6D2/F1 mice (Jackson

Laboratories, Bar Harbor MA) under open heart surgery as described in (7). Cells (4-10 x 10^4) were injected in a volume of 2-3 μl using a plastic syringe fitted with a 30 gauge needle.

<u>Histology.</u> Hearts were removed following cervical dislocation and cryoprotected in 30% sucrose, embedded and sectioned at 10 μm with a cryomicrotome as described (8). For hematoxylin and eosin (H and E) staining, sections were post fixed in acetone:methanol (1:1) and stained according to manufacturer's specifications (Sigma Diagnostics, St. Louis MO). For immuno-histology, unfixed sections were reacted with polyclonal rabbit anti-T-Ag antibodies (either 161-T, see (3) or 162-T) followed by horseradish peroxidase-conjugated goat anti-rabbit antisera (Boehringer Mannheim, Indianapolis IN), and visualized by diaminobenzidine reaction with nickel enhancement as described in (9). Monoclonal antibodies against the common leukocyte antigen (CD45; antibody M1/9.3HL, Boehringer Mannheim) and against the macrophage Mac-1 antigen (CD11b; antibody M1/70HL, Boehringer Mannheim) were used to monitor intra-cardiac graft rejection. The Mac-1 antibody has 75-90% cross reactivity with lymphocytes. After treatment with primary antibody, sections were incubated with horseradish peroxidase-conjugated rabbit anti-rat antisera (Boehringer Mannheim), and visualized by diaminobenzidine reaction with nickel enhancement. For [^{3}H]-thymidine incorporation, mice were given a single bolus injection of isotope (400 μCi at 28 Ci/mM, Amersham, Arlington Heights IL) and eighteen hours later sacrificed by cervical dislocation. The heart was removed, cryoprotected in 30% sucrose, embedded and sectioned with a cryomicrotome. Sections were post-fixed in methanol:acetone (1:1), stained with H and E, and a thin layer of photographic emulsion (Ilford L.4, Polysciences,

Warrington PA) diluted 1:1 with distilled water was applied. Sections were exposed for 5-7 days at 4°C, and developed in Kodak D-19 at 20°C for 4 minutes, washed with distilled water for 1 minute, fixed in 30% sodium thiosulfate for 10 minutes, and washed in distilled water.

Electron Microscopy (EM). Tissue blocks were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) and post-fixed in 2% osmium tetroxide (Stevens Metallurgical Corp., New York NY). All other EM chemicals were obtained from Ladd Research Industries, Inc. (Burlington VT). Tissue was stained en bloc with 2% uranyl acetate in pH 5.2 maleate buffer (0.05 M), dehydrated, and embedded in Ladd LX-112. Grafts were located using 1μm sections stained with toluidine blue. After trimming, the block was thin sectioned, and stained with uranyl acetate and lead citrate. Specimens were viewed on a Phillips 400 transmission electron microscope.

Electrocardiogram (ECG) Analyses. For surface ECG records, mice were anesthetized (2.5% Avertin,0.015 ml/g body weight, IP, Fluka Chemicals, Lake Ronkomkoma NY), surface electrodes were placed in the standard lead 1 position, and ECGs were recorded with a Narco Biosystems (Houston TX) high gain amplifier coupled to an A/D converter (Coulbourn Instruments, Lehigh Valley PA).

Plasma Enzyme Assay (PEA). For lactate dehydrogenase (LDH) isoform assay, plasma was isolated by retro-orbital sinus bleeds under anesthesia (2.5% Avertin,0.015 ml/g body weight, intraperitoneally (IP)). Plasma was fractionated on 1% agarose gels (CK Isoenzyme electrophoresis system, CIBA-Corning Diagnostics, Corning N.Y.) and the LDH isoforms visualized by a TNBT-Formazan histochemical assay (LDH Assay Kit, Sigma Diagnostics, St. Louis MO).

B.   RESULTS

In these studies, AT-1 cardiomyocytes (derived from transgenic animals that expressed the T-Ag oncoprotein in the heart) were injected directly into the myocardium of syngeneic mice and the viability of the grafted material was assessed.   To facilitate localization of the injection site in preliminary experiments, AT-1 cardiocytes were incubated briefly with BODIPY prior to grafting.   BODIPY is a nontoxic glutathione reactive dye which permits fluorescent tracking of living cells.   The graft site was easily visualized by fluorescence microscopy using a FITC cube.   Subsequent experiments did not utilize BODIPY.

Fifty percent (14/28) of the animals receiving AT-1 cardiomyocyte injections developed intra-cardiac grafts. In most instances, the grafts were neither encapsulated nor surrounded by scarred myocardium.   At the level of light microscopy, grafted AT-1 cardiomyocytes were observed directly juxtaposed with host cardiomyocytes. The identity of the AT-1 cardiomyocytes was confirmed by immuno-peroxidase assay using an anti-T-Ag antibody primary antibody (162-T) followed by a horseradish peroxidase conjugated secondary antibody.   Specificity of the anti-T-Ag antibody has been established previously (10, 11).   Black precipitate to was observed over cardiomyocyte nuclei in the graft but not in the host myocardium, confirming that the graft was comprised of AT-1 cardiomyocytes.   Similar results were obtained with other anti-T-Ag antibodies, and no signal was observed in the absence of primary antibody.

Viable AT-1 cardiomyocytes were observed at least as long as four months post-implantation.   During this period, some degree of graft proliferation occurred;

^{3}[H]-thymidine incorporation analyses detected DNA synthesis in the grafted cells. Ten percent of the AT-1 cardiomyocyte nuclei were synthesizing DNA as evidenced by isotope incorporation into the nucleus. However, the rate was appreciably less than that observed for cultured AT-1 cardiomyocytes, where 50% of the cells synthesized DNA following a similar ^{3}[H]-thymidine pulse. In several instances, the grafted AT-1 cardiomyocytes were localized within the subpericardial space.

Immunohistologic experiments were employed to determine if the intra- cardiac grafts were subject to chronic rejection. Grafts older than one month failed to react with antibodies specific for mouse leukocytes; signals observed in blood vessels located on the same section provided a positive control for the experiment. Similarly, an antibody which detects mouse macrophages and lymphocytes did not react with the intra-cardiac graft; once again positive signal was observed in a blood vessel located on the same section. Collectively, these results indicate the absence of chronic graft rejection by the syngeneic hosts. This result is supported by the observation that cyclosporine treatment (50 mg/kg body weight, administered intraperitoneally daily) did not influence significantly the frequency of intra-cardiac grafting (50% success rate, n=6). Sex of the host animal also did not appear to influence significantly the rate of graft formation (46% success rate in males,- n=13; 53% success rate in females, n=15). The frequency of grafting was similar in animals examined at early time points (1-40 days post-grafting, 47%, n=15) as compared to those examined at later time points (40-120 days post-grafting, 54%, n=13). Finally, similar frequencies of intra-cardiac grafting were observed when cells were delivered to either the left ventricular free wall or the apex of the heart.

Electron microscopic analysis of the AT-1 cardiomyocyte grafts confirmed the absence of encapsulation. High power views reveal d well-dev loped junctional complexes between adjacent cells within the graft. Graft cardiomyocytes contained numerous polyribosomes and the dedifferentiated myofibrillar ultrastructure typical of AT-1 tumors in vivo (6). Electron-dense secretory granules were also observed in the AT-1 cardiomyocyte grafts, as would be expected for myocytes of atrial origin. Host cardiomyocytes bordering the grafts had normal ultrastructure with well-formed sarcomeres. Although only a thin basement membrane separated AT-1 and host cardiomyocytes, no junctional connections between these two cell types were observed.

Surface electrocardiograms were performed to determine if the presence of AT-1 cardiomyocyte grafts influenced the autonomic rhythm. No appreciable differences were observed between records from sham animals and those which harbored grafts. In each case, the experimental animals exhibited normal sinus rhythm, with an anesthetized heart rate of approximately 400 beats per minute. Normal P-QRS coupling was maintained, indicating that the grafted AT-1 cardiomyocytes did not act as an ectopic pacemaker. This latter result is important in light of the observation that AT-1 cardiomyocytes exhibit spontaneous electrical activity both in vivo (12) and in culture (3). The absence of overt arrhythmia also indicated that graft-induced myocardial remodeling was not associated with the generation of significant circus rhythms.

In addition to surface ECG, plasma LDH levels were assessed in mice carrying AT-1 cardiomyocyte grafts. The presence of the cardiac LDH isoform in the circulation is a well established hallmark of myocardial infarction. No

cardiac LDH (isoform-1) was apparent in mouse plasma prior to grafting. After the introduction of AT-1 cardiomyocytes, there was a transient appearance of th cardiac isoform in the plasma, which most likely refl cted damage to the host myocardium as well as damaged AT-1 cardiomyocytes. A transient increase in plasma skeletal LDH isoform was also observed following grafting surgery, presumably reflecting damage caused by the trans-thoracic incision. The plasma LDH profiles returned to normal by 7 days post-implantation. Thereafter, the plasma LDH profiles remained normal despite the presence of grafts.

EXAMPLE 2
Generation of Stable C2C12 Myoblast Grafts

A.   METHODS

C2C12 Cell Culture and Myocardial Grafting Protocol.
C2C12 myoblasts were obtained from ATCC. Cells were maintained in the undifferentiated state by culturing at low density in high glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 20% fetal bovine serum, 1% chicken embryo extract, 100 units/ml penicillin and 100 μg/ml streptomycin. For some studies, myogenic differentiation was induced by culturing in DMEM supplemented with 2% horse serum and antibiotics. Immediately before injection, myoblasts were harvested with trypsin, washed three times with serum free DMEM and directly injected into the ventricular myocardium of adult syngeneic C3Heb/FeJ mice (Jackson Laboratories) under open heart surgery as described in (7). Cells ($4-10 \times 10^{4}$) were injected in a volume of 2-3 μl using a plastic syringe fitted with a 30 gauge needle.

Histology. Hearts were removed, cryoprotected, embedded and sectioned as in Example 1. H and E staining and

[^{3}H]-thymidine incorporation assays were also conducted as in Example 1. For immunohistology, methanol fixed sections (-20°C, 10 min.) were reacted with the monoclonal anti-skeletal myosin heavy chain antibody (MY-32, Sigma Chemical Corp.) followed by rhodamine-conjugated sheep anti-mouse IgG F(ab')$_2$ fragment (Boehringer Mannheim), and visualized by epifluorescence.

Electron Microscopy. EM was performed as in Example 1.

Electrocardiogram Analyses. ECG anlyses were performed as in Example 1.

Plasma Enzyme Assay. PEA was performed as in Example 1.

B. RESULTS

Several myoblast cell lines are known which, as exemplified by C2C12 cells, have the capacity to differentiate into myotubes in culture (13). C2C12 myoblasts were derived from cultured explants of injured thigh muscle of C3H mice. When maintained in serum-rich media, the myoblasts proliferate rapidly and retain an undifferentiated phenotype. However, when cultured in serum-poor media myogenic differentiation is induced. The C2C12 cells withdraw from the cell cycle and fuse, thereby forming multinucleated myotubes. Myogenic differentiation is also induced, as evidenced by the appearance of numerous muscle-specific gene products. Thus, in this model proliferation and myogenic differentiation are mutually exclusive (14). Myoblast differentiation in vitro is thought to mimic satellite cell mediated myofiber regeneration in vivo.

Myoblasts were injected directly into the myocardium of syngeneic C3Heb/FeJ mice and the viability of the

grafted material was assessed. One hundred percent (13/13) of the mice receiving intra-cardiac implants of C2C12 myoblasts developed grafts in the heart. Viable grafts were observed as long as six months post-implantation (this was the last time point assayed). In all instances, the grafted material was not encapsulated. The differentiated status of the grafted C2C12 cells was determined by immunohistological assay with an anti-myosin heavy chain antibody (MY-32). This antibody does not react with myoblasts nor with cardiac myosin heavy chain. Although differentiated C2C12 cells were observed in every heart receiving myoblast injections, the grafting efficiency of individual cells was not determined. As an additional control, hearts bearing AT-1 intra-cardiac grafts (see Example 1) were examined with the MY-32 antibody. No staining was observed, thereby ruling out the possibility that the signal seen in the C2C12 grafts was due to skeletal myosin heavy chain induction in host cardiomyocytes.

Example 1 above demonstrates that AT-1 cardiomyocytes form stable grafts in syngeneic myocardium. However, the observation that these cells retained the capacity for proliferation in vivo raised the possibility that sustained cell division might be required for successful intra-cardiac grafting. The proliferative status of the C2C12 grafts was therefore examined. Virtually no DNA synthesis (as assessed by tritiated thymidine incorporation) was observed, indicating that the majority of the grafted C2C12 cells had indeed withdrawn from the cell cycle. Examination of serial sections indicated that less than 0.1% of the cells in or near the grafts were synthesizing DNA. This result most likely reflects fibroblast proliferation during the remodeling process. As with the AT-1 grafts, immunohistological analyses of C2C12 grafts failed to detect macrophage,

inflammatory leukocyte or lymphocyte infiltration at two months post-implantation, indicating the absence of chronic graft rejection by the syngeneic hosts.

At the level of light microscopy, the C2C12 intra-cardiac grafts exhibited cellular heterogeneity with both H and E and MY-32 immunofluorescence staining. Electron microscopic analyses were employed in an effort to further characterize the cellular make-up of the C2C12 grafts. Toluidine-stained 1 μm sections were surveyed at 100 μm intervals to locate graft sites for EM analysis. Once localized, thin sections were prepared from the block. Cells with morphology typical of skeletal myocytes were observed throughout the graft. Abundant mitochondria localized between well developed sarcomeres were readily detected. Prominent Z bands and thick and thin filaments were observed. Occasionally, expanded t-tubules and ruffled cell membranes were detected in the grafted myocytes. In addition to well developed myocytes, a second less differentiated cell type was observed in C2C12 grafts. Most notably, these cells exhibited a large nucleus to cytoplasm ratio, with a prominent band of heterochromatin at the nuclear periphery. Moderate amounts of centrally located heterochromatin were also detected. Limited rough endoplasmic reticulum and few mitochondria were observed in these cells. Similar ultrastructural characteristics have been ascribed to satellite cells in vivo and in culture (15, 16).

Two studies were initiated to assess any deleterious effects of C2C12 intra-cardiac grafts on host heart function. In the first study, surface electrocardiograms failed to detect any appreciable differences between records from control and experimental mice. All animals examined had normal P-QRS coupling, and exhibited normal sinus rhythm with an anesthetized heart rate of

approximately 400 beats per minute. These data indicate
that the intra-cardiac myoblast grafts did not induce
overt cardiac arrhythmias. In the second study, plasma
LDH levels were monitored in graft-bearing animals. The
presence of the cardiac LDH isoform in the circulation is
a well established hallmark of myocardial infarction. The
cardiac-specific LDH isoforms (isoforms 1, 2, and 3) were
not observed in plasma prior to grafting. Immediately
after grafting, an increase in the cardiac isoforms was
observed in plasma, which most likely reflected damage to
the host myocardium. A transient increase in the plasma
skeletal LDH isoform (isoform 5) was also observed,
presumably reflecting damage caused by the trans-thoracic
incision. Plasma LDH profiles returned to normal by 7
days post-implantation.

EXAMPLE 3
Generation of Stable Fetal Cardiomyocyte Grafts

A.   METHODS

Cardiomyocyte Cell Culture and Myocardial Grafting
Protocol. Transgenic mice were generated which carry a
fusion gene comprised of the α-cardiac myosin heavy
chain (MHC) promoter and a modified ß galactosidase (nLAC)
reporter. To generate the MHC-nLAC transgenic mice,
MHC-nLAC insert DNA (see Figure 1) was purified by
absorption onto glass beads, dissolved at a concentration
of 5 µg/ml, and microinjected into the nuclei of one cell
inbred C3H3B/FeJ embryos according to established
protocols (17). Polymerase Chain Reaction (PCR) analysis
was employed to identify founder animals and to monitor
transgene segregation. The sense strand primer 5'-
GGTGGGGGCTCTTCACCCCCAGACCTCTCC-3' was localized to the MHC
promoter and the antisense strand primer
5'-GCCAGGGTTTTCCCAGTCACGACGTTGT-3' was localized to the

nLAC reporter. PCR analyses were as described in (18).
The MHC promoter consisted of 4.5 kb of 5' flanking
sequence and 1 kb of the gene encompassing exons 1 through
3 up to but not including the initiation codon. The nLAC
reporter was modified so as to carry both a eukaryotic
translation initiation site and the SV40 nuclear
localization signal (19). The mP1 sequences carried an
intron, as well as transcriptional termination and
polyadenylation signals from the mouse protamine 1 gene.

For preparations for examination of ß galactosidase
(ßGAL) activity and DAPI epifluorescence, transgenic
animals were heparinized (10,000 U/kg IP) prior to
sacrifice by cervical dislocation. Hearts were placed in
a beaker of gassed (95% O_2, 5% CO_2) KHB buffer (105 mM
NaCl, 20 mM $NaHCO_3$, 3.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM
$MgSO_4$, 0.01 mM $CaCl_2$, 1 mM mannitol, 10 mM taurine, 10
mM dextrose, 5 mM Na-pyruvate). Hearts were then hung by
the aorta and perfused with gassed KHB (0.5 ml/min at
37°C) containing 2.5 mM EGTA for five minutes, followed by
0.17% collagenase (Type I, Worthington Biochemical,
Freehold NJ) in KHB. Hearts were perfused until flaccid
and the ventricles were minced with scissors and isolated
cells obtained by triturating with a Pasteur pipette.
After at least one hour of formalin fixation, suspensions
were filtered and smeared onto positively charged slides
(Superfrost Plus, Fisher, Pittsburgh PA), and allowed to
dry.

For isolation of single cells for injection, females
with 15 day embryos (onset of pregnancy determined by
vaginal plugs) were sacrificed by cervical dislocation.
Embryos were removed, decapitated, and hearts were
harvested under PBS, and ventricles and atria were
separated. Transgenic ventricles (identified by cardiac
ßGAL activity) were digested in 0.1% collagenase

(Worthington) in DPBS (Dulbecco's Phosphate Buffered
Saline, Sigma) for 45 minutes, and were triturated with a
Pasteur pipette in PC-1 medium (Ventrex, Coons Rapids MN)
with 10% FBS, resulting in a suspension of single cells.

Immediately after isolation, embryonic cardiomyocytes
were washed three times with DPBS and directly injected
into the ventricular myocardium of syngeneic mice (Jackson
Laboratories) under open heart surgery as in Example 1.
$1-10 \times 10^4$ cells were injected in a volume of 2-3 μl
using a plastic syringe fitted with a 30 gauge needle.

Histology. For H and E, X-GAL, immunohistology and
thymidine analyses, hearts were removed following cervical
dislocation and cryoprotected in 30% sucrose, embedded and
sectioned at 10 μm with a cryomicrotome as in Example 1.
H and E staining, monitoring for intra-cardiac graft
rejection, and assay for [^{3}H]-thymidine incorporation
were also conducted as in Example 1. To assay ßGAL
activity, sections were hydrated in PBS, post-fixed in
acetone:methanol (1:1) and then overlaid with mixture
containing 1 mg/ml X-GAL
(5-bromo-4-chloro-3-indolyl-ß-D-galactoside), 5 mM
potassium ferricyanide, 5 mM potassium ferrocyanide and 2
mM magnesium chloride in PBS. Positive staining is
indicated by the appearance of a blue chromophore. After
treatment with primary antibody, signal was visualized by
an avidin-biotin (ABC) kit (Vector Labs, Burlingame CA).
The heart was processed as described above, and sections
were post-fixed in methanol:acetone (1:1), stained with H
and E, and coated with a thin layer of photographic
emulsion (Ilford L.4, Polysciences) diluted 1:1 with
distilled water. Sections were exposed, developed,
washed, fixed and washed as in Example 1. X-GAL staining
of single cell preparations was as described above. For
visualization of nuclei in single cell preparations,

slides were stained with DAPI in PBS (0.28 µM, three min. at room temperature, Boehringer Mannheim), washed three times in PBS, and wet-mounted in 2% propyl gallate dissolved in glycerol. To obtain coronal heart sections, mice were sacrificed by cervical dislocation, hearts were harvested and perfused on a Langendorff apparatus with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After immersion fixation overnight in the same buffer, 200 µm coronal sections were made with a vibratome (Campden, London, United Kingdom). To localize the graft, sections were pooled and stained for ßGAL activity with X-GAL as described above.

Electron Microscopy. MHC-nLAC embryonic grafts were localized in coronal heart sections as described above. After trimming, the tissue was post- fixed in 2% osmium tetroxide (Stevens Metallurgical Corp., New York NY). Tissue was then dehydrated and embedded in Ladd LX-112 (Ladd Research Industries). Grafted areas were further trimmed, thin sectioned, and stained with uranyl acetate and lead citrate. Specimens were viewed on a Phillips 400 transmission electron microscope as in Example 1.

Electrocardiogram Analyses. ECG analyses were conducted as in Example 1.

B.   RESULTS

Transgenic mice generated as above carried a fusion gene comprised of the MHC promoter and a nLAC reporter. nLAC carries the SV40 nuclear transport signal, which results in the accumulation of ß galactosidase activity in the nucleus of targeted cells. Four transgenic lineages were produced, and two (designated MHC-nLAC-2 and MHC-nLAC-4) were selected for further analyses. To ensure

that the MHC-nLAC transgene would provide a suitable cell
lineage marker, ß galactosidase (ßGAL) activity was
assessed in transgenic cardiomyocytes.   Single cell
preparations generated by retrograde collagenase perfusi n
were examined simultaneously for ßGAL activity and DAPI
epifluorescence.   99.0 ± 0.45% (n=400) of the transgenic
cardiomyocyte nuclei expressed ßGAL, whereas no ßGAL
activity was detected in noncardiomyocytes.   In addition,
no nuclear ßGAL activity was detected in nontransgenic
control cardiomyocytes.

Single cell suspensions were prepared by collagenase
digestion of hearts harvested from embryonic day 15
transgenic mice.   Greater than 95% of the cardiomyocytes
isolated by this technique were viable as evidenced by dye
exclusion assay.   Cardiomyocytes were delivered to left
ventricular free wall of syngeneic nontransgenic animals.
Grafted cardiomyocytes were readily and unambiguously
identified by virtue of the nuclear ßGAL activity encoded
by the MHC-nLAC transgene.   Grafted cardiomyocytes were
frequently observed at sites distal to the point of
delivery; it presently is not clear if this distribution
of grafted cells reflects cardiomyocyte migration or
passive diffusion along dissection planes produced by the
injection process.   Approximately 50% (7/13) of the
animals receiving intra-cardiac injections of embryonic
cardiomyocytes developed grafts.   This frequency of
successful graft formation is likely to increase as cell
preparation and implantation protocols are optimized.

Light microscopic analyses of H and E stained
sections processed for ßGAL activity indicated that
grafted cardiomyocytes (blue nuclei) were juxtaposed
directly with host cardiomyocytes (purple nuclei).
Additional H and E analyses failed to detect significant
graft encapsulation.   The observed proximity of graft and

host cardiomyocytes and absence of encapsulation are prerequisites for successful coupling between the two cell types.

Consecutive sections of a 19 day old intra-cardiac graft were processed for H and E, ßGAL activity, and macrophage and leukocyte immunoreactivity. No evidence for graft rejection was observed, despite the fact that the animals were not immune suppressed. As a positive control for the immunohistology, grafts of incompatible MHC haplotype were produced; graft rejection was clearly evident in these hearts. Tritiated thymidine uptake analyses indicated that only 0.6% (n=156) of the ßGAL-positive nuclei were synthesizing DNA, although noncardiomyocyte DNA synthesis was apparent. Since the embryonic day 15 donor cells were still mitotically active when grafted (labeling index of ca. 29%), the exceedingly low level of DNA synthesis observed in ßGAL positive cells at 19 days post-grafting suggested that the MHC-nLAC embryonic cardiomyocytes had undergone terminal differentiation.

The juxtaposition of graft and host cardiomyocytes observed by light microscopic analyses prompted a determination whether direct intercellular coupling could be detected between the two cell types. The X-GAL reaction product is an electron-dense precipitate which can be detected by transmission electron microscopy (TEM, see 19). Vibratome sections from glutaraldehyde perfusion-fixed hearts were stained for ßGAL activity, and grafted regions thus identified were trimmed and embedded for TEM. ßGAL positive nuclei were readily observed by light microscopic analysis of 1 μm sections. The X-GAL reaction product had a perinuclear appearance due to a slight degree of nuclear leaching which occurred during the embedding process. The same groups of cardiomyocytes

were identified by TEM analysis of a consecutive thin
section. Host cardiomyocytes, which were not readily
identified in the light micrographs due to the absence of
perinuclear ßGAL activity, were observed by lectron
microscopy to be juxtaposed with the grafted cells.
Numerous junctional complexes were present between the
host and graft cardiomyocytes, indicating a high degree of
intercellular coupling. Many examples of intercellular
coupling between host and graft cardiomyocytes were
observed throughout the grafted regions. Importantly,
intercellular connections could be traced from ßGAL
positive cardiomyocytes through numerous host cells, thus
demonstrating that grafted cardiomyocytes could be
participating in a functional syncytium.

In addition to documenting the presence of abundant
intercellular coupling between grafted and host
cardiomyocytes, the TEM analyses revealed that the grafted
cardiomyocytes were highly differentiated. Normal
characteristics of adult cardiomyocytes were observed
including myofibrillae forming complete sarcomeres,
numerous junctional complexes between cells and abundant
mitochondria. Indeed, aside from the presence of the
X-GAL reaction product, grafted cardiomyocytes were
indistinguishable from host cells. Further, binucleated,
ßGAL positive cells could be detected in the intra-cardiac
grafts. Because binucleation is a characteristic of adult
rodent cardiomyocytes, this observation further supports
that the grafted cardiomyocytes have undergone terminal
differentiation.

Surface ECG recordings were employed to determine if
the presence of coupled embryonic cardiomyocyte grafts
negatively influenced host heart automaticity. ECG traces
from graft-bearing animals were indistinguishable from
sham operated controls, and exhibited P and QRS complexes

typical for mice. There was no evidence for cardiac arrhythmia in graft-bearing animals, despite the presence of a high degree of intercellular coupling between grafted and host cardiomyocytes.

EXAMPLE 4
Preparation of Substantially Pure Cardiomyocyte Culture

Embryonic stem cells were genetically modified in a manner enabling the production of a substantially homogeneous population of non-immortalized cardiomyocytes. The parental ES cell line (D3) was cotransfected with a pGK-HYG (hygromycin) plasmid and a plasmid containing a MHC-neo^r gene. The pGK-HYG plasmid provides selection for transfected ES cells, while the mMHC-neo^r gene facilitates a second round of selection on differentiated cells: incubation in the presence of G418 eliminates non-cardiomyocytes (that is, cells in which the MHC promoter is not active).

Stably tranfected ES cells were selected by growth in the presence of hygromycin. The plasmids were linearized and introduced into the stem cells via electroporation at 1180 µFarad, 220 volts. The transfected cells were maintained in DMEM supplemented with 10% preselected FBS, 0.1 mM ß-mercaptoethanol, nonessential amino acids, PenStrep and LIF, and transformants selected by the addition of hygromycin into the medium. Co-transfectants were then identified by PCR analysis specific for both transgenes. The transfections produced a cell line, designated 9A, which carries both transgenes.

Cardiogenesis was induced in 9A ES cells by plating 2 x 10^6 cells onto uncoated 100 mm bacterial petri dishes in the absence of LIF. After 8 days in culture, numerous patches of cells exhibiting spontaneous contractile

activity (i.e. cardiomyocytes) were observed. At this
point, G418 was added to the media, and the cells
incubated for an additional 9 days. During this treatment
it was apparent that many of the non-cardiomyocytes were
being killed by the G418. Importantly, the G418 had no
discernible effects on the cardiomyocytes, which retained
their spontaneous beating activity throughout the course
of the experiment.

After a total of 9 days of G418 selection, the
surviving cells were dissociated with collagenase and
trypsin, and then replated onto fibronectin coated
microscope slides. The cells were cultured an additional
24 hours to allow them to recover from passage, and then
fixed for immunocytologic analysis. Cells were reacted
with MF20, a monoclonal antibody which recognizes
sarcomeric myosin. Cells were then individually counted
for the presence or absence of sarcomeric myosin, a marker
for cardiomyocytes. The results were as follows:

Total number of cells counted:	794
Number of MF20+ cells:	791
Number of MF20- cells:	3*
Percent cardiomyocytes:	99.6%

*The 3 cells which did not stain with MF20 may still
have been cardiomyocytes, since this antibody will
not react with monomeric myosin.

It was thus demonstrated that the expression of drug
(neomycin) resistance under a cardiac-specific promoter
enables the selection of an essentially pure population of
ES derived cardiomyocytes in culture. Such populations
can be used to form myocardial grafts using procedures as
discussed in the Examples above.

EXAMPLE 5
Delivery of Protein via Graft

A. METHODS

C2C12 Cell Culture and transfection. C2C12 myoblasts (ATCC) were maintained in the undifferentiated state by culturing at low density in high glucose Dulbecco's Modified Eagle Media (DMEM)[1] supplemented with 20% fetal bovine serum, 1% chicken embryo extract, 100 units/ml penicillin and 100μg/ml streptomycin. For some studies, myogenic differentiation was induced by culturing in DMEM supplemented with 2% horse serum and antibiotics.

A fusion gene comprised of the metallothionein (MT) promoter driving a modified Transforming Growth Factor-Beta 1 (TGF-ß1) cDNA was obtained from Samuel and colleagues (20). Transcriptional activity of the metallothionein promoter can be regulated by modulating the heavy metal content of cell culture media. The TGF-ß1 cDNA carried site-directed mutations which resulted in the conversion of Cys^{223} and Cys^{225} to serines. This modification (described further in (21)) results in the elaboration of a TGF-ß1 molecule which is unable to form dimers, and consequently is not subject to normal post translational regulation. Cells expressing the modified cDNA constitutively secrete processed, active TGF-ß1 (20). The MT-TGF fusion gene was introduced into C2C12 myoblasts by calcium phosphate transfection; stable transfectants were selected by virtue of co-transfection with an SV40-neo[r] transgene. Four independent clones were isolated, and presence of the transgene was confirmed by Southern blot analysis. The relative levels of TGF-ß1 expression in the different clonal cell lines was initially assessed by Northern blot analysis, and one line, designated C2(280), was utilized for subsequent experiments.

Myocardial Grafting Protocol. The grafting protocol
was as described in Example 1. Fourteen days
post-surgery, graft bearing animals were given heavy metal
(25 mM $ZnSO_4$ in drinking water). Zinc treatment was
continued until the termination of the experiment (1-4
weeks).

Histology. For paraffin sections, hearts were fixed
in 10% neutral buffered formalin, dehydrated through
graded alcohols, and infiltrated with paraffin. Tissue
blocks were then sectioned at 6μm. H and E staining was
performed directly after sectioning according to
manufacturer's specifications (Sigma Diagnostics).

For [^{3}H]-thymidine incorporation, mice were given a
bolus and sacrificed as in Example 1. The heart was
removed and processed for paraffin embedding as described
above. Autoradiography was likewise conducted as in
Example 1.

B. RESULTS

Expression of recombinant TGF-ß1 in response to heavy
metal induction was examined in C2(280) myoblasts and
myotubes. Transgene transcripts (1.8 kb) were readily
distinguished from those originating from the endogenous
TGF-ß1 gene (2.5 kb) by Northern blot analysis. Addition
of heavy metal to the culture media resulted in a marked
increase of recombinant TGF-ß1 transcripts in C2(280)
myoblasts and myotubes. As indicated above, modified
TGF-ß1 expressed by C2(280) cells should have constitutive
biological activity. To directly test this, conditioned
media from C2(280) myoblasts and myotubes was examined by
growth inhibition assay.

C2(280) myoblasts were used to produce intra-cardiac grafts in syngeneic C3Heb/FeJ mice. The presence of grafts was readily detected in H and E stained sections. 100% (n > 50) of the animals receiving intra-cardiac injections of C2(280) cells went on to develop grafts. Interestingly, H and E analysis suggested that the C2(280) grafts were somewhat less differentiated as compared to those produced with unmodified C2C12 cells. This result was confirmed by immunohistologic analysis with a monoclonal antibody which recognizes skeletal myosin heavy chain.

C2(280) graft transgene expression was assessed by immunohistology with an anti-TGF-ß1 antibody; TGF-ß1 expression was readily detected in C2(280) grafts. As a negative control, TGF-ß1 expression was assessed in grafts produced by C2C12 myoblasts. As expected, the relative levels of TGF-ß1 expression were markedly reduced in C2C12 grafts as compared to C2(280) grafts.

TGF-ß1 is a well known angiogenic factor. ^{3}H-thymidine incorporation analyses in vascular endothelial cells was therefore assessed to determine if an enhanced angiogenic response occurred in grafts expressing the MT-TGF transgene. DNA synthesis in vascular endothelial cells was readily apparent in C2(280) grafts under administration of a single bolus injection of ^{3}H-thymidine (H-THY). In contrast, vascular endothelial DNA synthesis was markedly reduced in non-transfected C2C12 grafts (Table 1). To rule out the possibility that the angiogenic responses was due solely to graft mass, ^{3}H-thymidine incorporation was compared between similar size and aged C2C12 and C2(280) grafts (Table 1). A marked increase in the number of vascular endothelial cells synthesizing DNA was apparent in all of the

analyses.   Finally, the thymidine incorporation assay also
revealed that a percentage of the grafted myoblasts
continued to proliferate.   This observation is consist nt
with the known inhibitory effect of TGF-ß1 on
myodifferentiation, and most likely accounts for the
undifferentiated appearance of the C2(280) grafts.

TABLE 1
TGF-β1 Delivery and
Vascular Endothelial DNA Synthesis

Time Post Zn Induction		C2C12 TGF-β1(-)	C2(280) TGF-β1(+)
1 Week	Total # ^{3}H-THY+ Endothelial Cells	0	8
	Total # of Vessel Sections Counted	35	17
	# Synthetic Cells/ Vessel Section	0.0 ± 0.00	0.46 ± 0.036
2 Weeks	Total # ^{3}H-THY+ Endothelial Cells	1	7
	Total # of Vessel Sections Counted	18	21
	# Synthetic Cells/ Vessel Section	0.06 ± 0.056	0.34 ± 0.052

While the invention has been illustrated and described in detail in the foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been described and that all modifications that come within the spirit of the invention are desired to be protected.

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